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Establishment of Efficient Regeneration System from Leaf Discs in Long Pepper an Important Medicinal Plant (*Piper longum* L.)

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Abstract

Cultivation of *Piper longum* L. till recently was not very common; still it is extensively collected from the wild, threatening the very existence of the plant. Hence, it is alarming that there is an urgent need not only cultivate this plant but also develop conservation strategies. Conventional propagation is overwhelmed with problems of poor seed viability low percentage of germination, scanty and delayed rooting of vegetative cuttings indicating there is a need for alternative propagation methods. *In vitro* technique is an alternative approach to solve the problem. Therefore, the current research was aimed at developing a promising *in vitro* mass propagation protocol for *Piper longum* L. using leaf as explant. Murashige and Skoog medium was used throughout the experiment. For callus induction, MS medium supplemented with alone or in combination of IAA (1 to 2 mg/l) and BAP (1 to 2 mg/l) were used. Shoot induction was undertaken on MS medium supplemented with different concentrations and combinations of Kinetin and BAP (1 to 3 mg/l). Emerged shoots were transferred onto elongation medium supplemented with 2 mg/l Zeatin and 1 mg/l GA_3. *In vitro* rooting was achieved on ½ MS medium+NAA 1 mg/l. Accordingly, the highest calli were induced on MS+1 mg/L IAA+1 mg/L BAP. Among the various treatments, the maximum percentage (91.50 ± 3.54) *in vitro* shooting was observed on MS+2 mg/L BAP+1 mg/L Kinetin. *In vitro* rooted shoots were successfully acclimatized in the greenhouse conditions. Therefore, it is possible to deduce that the current protocol is promising for *in vitro* mass propagation of *Piper longum* L. to solve the reproduction and cultivation problem of the plant.

Keywords: Leaf segments; Callus induction; Multiple shoots; Callus necrosis; Regeneration

Abbreviations: IAA: Indole 3 acetic acid; BAP: 6-Benzyl amino purine; NAA: Naphthalene Acetic Acid; GA_3: Gibberellic acid

Introduction

Plants have been used for medicinal application ever since man began caring for his body and health. For centuries, the world has depended on the valuable properties of plant as a source of healing. Ayurveda, Siddha, Unani and Homeopathy continue to depend predominantly on medicinal and aromatic plants as raw material for the formulation of drugs. Medicinal Plants have attracted attention of not only professionals from various systems of medicine, but also the scientific community belonging to different disciplines. *Piper longum* L. of family Piperaceae commonly known as long pepper is a unisexual perennial climber indigenous to the hotter parts of India and grows wild in the ever green forests of Western Ghats [1]. The compound of medicinal interest in *Piper longum* L. is present in the female spike (inflorescence) and leaf possesses antdiabetic, antiplatelet, antiulcer, antifertility, cardiotonic, antitumour, antimutagenic, hypotensive, respiratory depressant, anthelmintic activities. The principal pharmacological constituents are piperine and piplartin [2-4]. Cultivation of *Piper longum* L. till recently was not very common still it is extensively collected from the wild, threatening the very existence of the plant [5]. Hence it is alarming that there is an urgent need not only cultivate this plant but also develop conservation strategies. Conventional propagation is overwhelmed with problems of poor seed viability low percentage of germination and scanty and delayed rooting of vegetative cuttings indicating there is a need for alternative propagation methods [6]. Due to with increasing market demand efforts are being diverted towards systemic cultivation of *Piper longum* L. Since it is a shade loving creeper, majority of cultivators grow them as intercrop in coffee and coconut plantation. The present work on this important medicinal plant was undertaken to optimize multiple shoot propagation protocol using leaf disc as an explant.

Materials and Methods

Explant collection and culture establishment

Tender leaves were collected from mother plant grown under controlled conditions in green house by cutting a twig with a sharp sterile scalpel blade, before 8 AM in the morning at a time when it has the higher turgor pressure and immediately dipped in 3% bavistin for 10 min and then washed with tween 20 under running tap water followed by rinsing with sterile water. Blot dried on a filter paper for 5 min and transferred under laminar flow. The explants were trimmed (2 to 3 cm long) and surface sterilized with 0.1% mercury chloride for 3 min. This was followed by washing of the explants in 3 to 4 changes of sterile water. Further surface sterilized the explants with 1% sodium hypochlorite for 5 min and then rinsed five times with sterile water, each wash for 1 min. Surface sterilized explants were dried on sterile filter paper for 5 min. These surface sterilized leaf discs (2 to 3 cm long) were inoculated on culture medium under aseptic conditions for callus induction. Each treatment consists of 2 replicates and the experiment repeated 2 times.

Culture medium and conditions

Murashig and Skoog medium was used as basal medium for callus

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induction supplemented with alone IAA (1 to 2 mg/l) and BAP (1 to 2 mg/l) and in combination with various concentration and 100 mg/l ascorbic acid. The pH of the medium was adjusted to 5.8 before autoclaving at 15 psi (121°C) for 20 min. The culture medium was gelled with 0.8% PTC grade agar (Himedia). All the cultures were incubated at 25 ± 2°C with 14 hrs of photo period produced by cool white light with a light intensity of 200 to 400 fluxes. Subculture was done for every 12 days for preventing browning of tissue due to phenolic exudates. After 20 to 25 days callus was formed from the leaf discs. The induced callus was subcultured on to MS medium supplemented with alone BAP (1 to 3 mg/l) and kinetin (1 to 3 mg/l) and in combination with various concentration and 100 mg/l ascorbic acid for multiple shoot induction and incubated at 25 ± 2°C with 16 hrs of photo period produced by cool white light with a light intensity of 200 to 400 fluxes. Within 15 days, emerged shoot primordia were transferred onto shoot elongation medium composing MS medium+Zeatin 2 mg/l+GA_3 1 mg/l, and incubated at culture conditions of 25 ± 2°C with 14 hrs of photo period produced by cool white light with a light intensity of 200 to 400 fluxes. Well elongated shoots were rooted on ½ MS basal medium+glucose (15 g/l)+Ascorbic acid (100 mg/ l)+Agar (8 g/l) supplemented with NAA 1 mg/l. The well rooted plantlets were hardened and covered with polythene bags for acclimatization at green house conditions.

Results

All the leaf disc explants responded readily on MS medium supplemented with IAA and BAP alone and in combination with various concentrations for callus induction. However, 1 mg/l IAA and 1 mg/l BAP gave green, globular, compact callus in 95% of the cultures, and calli from these medium proliferated rapidly than other treatment combinations (Figure 1). ANOVA showed that the various concentration and combinations of BAP and Kinetin have very significant effect ($p<0.0001$ at $\alpha=5\%$) on shoot regeneration. Among the various concentrations and combinations, MS medium supplemented with 2 mg/l BAP and 1 mg/l kinetin showed the maximum (91.50 ± 3.54) shoot primordia induction frequency (Table 1) within 15 days that gave on average 20-25 shoots per callus (Figure 2). As can be reveled from Table 1, use of BAP (2 mg/l) and Kinetin (1 mg/l) alone induced a maximum of 44.50 ± 2.12 and 24.50 ± 2.12 shoots per callus respectively. Moreover, inclusion of these two cytokinins induced the highest (91.50 ± 3.54)) shoots per calli. Keeping BAP at 2 mg/l, a further

Figure 1: Callus induction from leaf discs, 1 mg/l IAA and 1 mg/l BAP.

BAP	Kinetin	Percent of Shoot Induction (Mean ± SD)
1	0	27.50 ± 2.12^{d}
1.5	0	29.00 ± 1.41^{d}
2	0	43.50 ± 2.83^{c}
2.5	0	44.50 ± 2.12^{c}
3	0	44.50 ± 3.53^{c}
0	1	21.00 ± 4.24^{e}
0	1.5	26.00 ± 0.00^{de}
0	2	24.50 ± 0.71^{de}
0	2.5	24.50 ± 2.12^{de}
0	3	21.50 ± 0.71
1	1	44.00 ± 2.83^{c}
1.5	1.5	46.00 ± 1.41^{c}
2	2	56.50 ± 3.54^{b}
1.5	1	61.50 ± 2.12^{b}
2	1	91.50 ± 3.54^{a}
CV (%)	**6.22**	
R^2	**99.08**	

Means in column with the same letter are not significantly different by Duncan's Multiple Range Test at $\alpha=5\%$ significant level.

Table 1: Effect of different concentration and combinations of BAP and Kinetin on *in vitro* shoot induction percentage.

Figure 2: Multiple shoot induction from callus, 2 mg/l BAP and 1 mg/l Kinetin.

increase of kinetin from 1 mg//l to 2 mg/l decreased the shoot induction frequency. Usable shoots were excised carefully without injury and transferred onto elongation medium supplemented with 2 mg/l Zeatin and 1 mg/l GA_3. All the shoots were elongated well in the elongation medium in jam bottles after two successful sub-culturing stages each 10 days. All the elongated shoots were transferred onto ½ MS medium supplemented with 1 mg/l NAA and excellent rooting was obtained without losing shoots. Interestingly, along with the roots, bulb like structure was observed at base of shoots. Excising the swelling portion and re-culturing on shoot induction medium produced multiple shoots. Well rooted plants were hardened in green house conditions and the survival rate is 100%.

Discussion

In plant culture, no two genotypes give similar response under a given set of culture conditions [7,8]. It often requires testing of various

type, concentration and mixture of the growth regulators during the development of a tissue culture protocol for a new plant tissue [9]. In the current study, among the various concentration and combinations of IAA and BAP tested, the maximum calli were induced on MS basal medium+glucose (30 g/l)+1 mg/l IAA+1 mg/l BAP. In culture establishment the major problem was bacterial contamination due to endogenous bacteria, and this was overcome using topmost and tender actively growing leaves explants. Phenolic exhudates causes browning and necrosis of explants as this was overcome by toting up the callus induction medium with ascorbic acid and frequent subculturing. Regarding multiple shoot induction, among the different concentration and combinations of BAP and Kinetin, the lowest (21.00 ± 4.24) shoot induction was seen when Kinetin (1 mg/l) alone was used while the highest frequency (91.50 ± 3.54) of shoots were induced when 2 mg/l BAP was included. This implies that inclusion of these cytokinins have synergic effect in promoting shoot induction. However, maintaining BAP at 2 mg/l and a further increase of Kinetin concentration will result in reduction of shoot induction frequency. From this it is possible to deduce that 2 mg/l BAP+1 mg/l Kinetin is optimal for maximum shoot induction of the plant. BAP induced multiple shoots from nodal explants of *Lawsonia inermis* [10]. Niranjan et al. [11] reported most multiple shoots in *Lagerstroemia indica* by supplementing with BAP. Similarly, Sarasan et al. [6,12] observed multiple shoots from the same plant on MS medium supplemented with BAP+Kinetin. Shoot elongation was achieved on MS basal medium+glucose (30 g/l)+Ascorbic acid (100 mg/ l)+Agar (8 g/l)+Zeatin 2 mg/l+GA_3 1 mg/l. Effective rooting was undertaken on ½ MS basal medium+glucose (15 g/l)+Ascorbic acid (100 mg/ l)+Agar (8 g/l) supplemented with NAA 1 mg/l.

Conclusion

Lack of a steady supply of good planting material is one of the bottle necks for the exploration of the potential of *Piper longum* L. *In vitro* mass propagation ensures quick availability of genetically uniform (true to type) diseases free planting materials within short period of time. In the present study, an effective protocol for subsequent *in vitro* plantlets multiplication from leaf disc explants was developed for this medicinally important plant. Accordingly, MS medium supplemented with 2 mg/l BAP+1 mg/l kinetin+3% Glucose is found to be the best combination for maximum (91.50 ± 3.54) multiple shoot induction frequency. Hence, this treatment combination is found to be optimal for *in vitro* multiplication of the plant to solve the commercialization and conservation bottle necks of this economically important medicinal plant.

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We have no conflict of interest.

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